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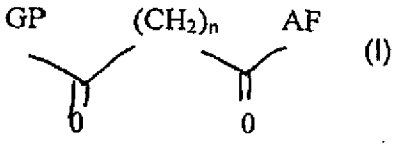

(57) Abstract: There is described a compound of the general formula (I): wherein GP is a glucose polymer, or a mixture of glucose polymers, and optionally salts thereof; AF is an antifungal agent; and n is an integer from 1 to 12. There is also described compositions containing this compound and methods of treatment relating thereto.

Dextrin- AmpB at 0.5 mg/ml

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Medicaments

This invention relates to a novel form of medicament, novel formulations comprising the medicament and novel methods of treatment.

Amphotericin is a polyene antibiotic which is active against both yeast-like and filamentous fungi, acting on sterols in the fungal cell membrane to cause an increase in permeability and leakage of cell constituents. Resistance to polyene antifungals in normally sensitive fungi, is virtually unknown. Orally delivered preparations of antifungals, such as amphotericin, are useful for the treatment of, *inter alia*, oral thrush and intestinal candidiasis and for the suppression of Candida in the GI tract which may, for example, be acting as a reservoir in recurrent vaginal candidiasis. Intravenous preparations of antifungals, such as amphotericin, are used for life-threatening systemic mycoses.

Amphotericin by intravenous infusion (e.g. Fungizone®) is used for the treatment of systemic fungal infections and is active against most fungi and yeasts. Amphotericin is given intravenously for severe systemic fungal infections (e.g., aspergillosis, cryptococcal meningitis, disseminated cryptococcosis), and is used in immunocompromised (for example, AIDS or transplantation) patients who are at particular risk of severe, disseminated fungal-type infections. Amphotericin was first shown to have activity against the leishmaniasis protozoan infection in the late 1950s, and is used to treat visceral and mucocutaneous leishmaniasis. Again activity is probably due to amphotericin's affinity for sterols.

It is highly protein bound and penetrates poorly into body fluids and tissues.

When given parenterally amphotericin is toxic and side effects are common. Close supervision is necessary and a test-dose is required. Typical side effects from parenteral administration include; anorexia, nausea and vomiting, diarrhoea, epigastric pain, febrile reactions, headache, muscle and joint pain, anaemia; renal

function disturbance (including hypokalaemia and hypomagnesaemia), renal toxicity; cardiovascular toxicity (including arrhythmias), blood disorders; neurological disorders (including hearing loss, peripheral neuropathy); abnormal liver function, rash, possible anaphylactoid reaction, and pain and thrombophlebitis at injection site

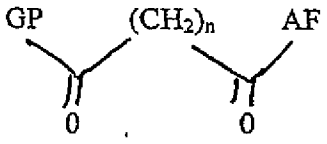
Lipid formulations of amphotericin (Abelcet®, AmBiSome®, and Amphocil®) are significantly less toxic but are expensive, for example, a 20 day dosage regime of AmBiSome® costs in the region of £12,000 (twelve thousand pounds sterling).

Therefore, current practice is that non-lipid formulations (e.g. Fungizone®) are tried first and, because of the cost implications, lipid-based formulations are used very reluctantly. The reduced toxicity of the lipid formulations has, however, led to its more frequent use as a leishmanicide.

Dextrins are known from European Patent Applications Nos 0 115 911 and 0 153 164 and are useful in peritoneal dialysis. When used in peritoneal dialysis, dextrins are generally administered into the peritoneal cavity as an aqueous solution.

We have now surprisingly found that certain antifungal agents, such as amphotericin, may be bonded to a glucose polymer, e.g. a dextrin polymer, to produce a novel and therapeutically advantageous compound.

Thus, according to the invention we provide a novel compound of the general formula I,



I

wherein GP is a glucose polymer, or a mixture of glucose polymers, and optionally salts thereof;

AF is an antifungal agent; and

n is an integer from 1 to 12.

Since protozoal infections such as Pneumocystis carinii are now thought possibly to be fungal infections, by the term antifungal used herein, it is intended to include antiprotozoal agents and anti-leishmaniasis agents.

According to a further feature of the invention we provide a pharmaceutical composition comprising an effective amount of a complex of an antifungal agent and a glucose polymer, or a mixture of glucose polymers and optionally salts thereof.

The antifungal agent is preferably an antifungal agent which is capable of bonding to a glucose polymer. Therefore, preferentially, the antifungal agent may possess at least one free amine group. Such antifungal agents include polyene antibiotics, such as amphotericin and nystatin.

In an especially preferred embodiment of the invention the antifungal agent is amphotericin, e.g. amphotericin B.

Although any conventionally known glucose polymers may be used, preferred glucose polymers or a mixture of glucose polymers, and optionally salts thereof, are those polymers described in European Patent Applications Nos 0 115 991 and 0 153 164. Salts include the anionic salts, e.g. sulphates. Glucose polymers which may hereinafter be referred to as dextrin, glucose dextrin or dextrin polymer are intended, on all occurrences, to include optionally salts thereof especially the sulphate.

The dextrin sulphate optionally would contain at most two sulphate groups per unit. All references herein to dextrin sulphate, dextrin 2 sulphate, or D-2-S are within the scope of the aforementioned definition.

Dextrin is a mixture of polymers of glucose and the glucose units may be substituted in one or more of the 2, 3 and 6 positions by sulphate groups.

A dextrin sulphate of use in the present invention may have up to 2, 3 and 6 positions occupied by sulphate groups.

A dextrin sulphate of use in the present invention may have up to two sulphate groups per glucose unit and preferred dextrin sulphates are those having about 1, or between 0.5 and 1.5, preferably up to 1.2, for example 1.1, sulphate groups per glucose unit. More preferably, the agent is the 2- or 6-sulphate of dextrin or a mixture thereof, most preferably dextrin-2-sulphate (D-2-S) that is dextrin wherein a substantial proportion of the sulphate groups are in the 2-position, preferably greater than 75%, more preferably greater than 90%, e.g. 94%.

Therefore, according to a preferred embodiment of the invention we provide a pharmaceutical composition as hereinbefore described wherein the glucose polymer comprises a glucose polymer mixture, said mixture including more than 15% by weight of glucose polymers of D.P. greater than 12, more preferably, more than 50% by weight of glucose polymers of DP greater than 12.

The mixture may contain from 50 to 90% by weight of glucose polymers of D.P. greater than 12. It is advantageous to use a mixture containing from 75 to 100%, preferably 90 to 100%, by weight of glucose polymers of D.P. greater than 12. The average molecular weight of the polymer mixture is preferably from 15,000 to 25,000, more preferably 18,000 to 22,000 (as determined by high pressure liquid chromatography). It is particularly desirable that the content of oligosaccharides in the glucose polymer mixture should be kept at a low level. The oligosaccharide

content of the glucose polymer mixture should be no higher than 10% by weight, the mixture containing from 90 to 100% by weight of glucose polymers of D.P. Thus the preferred glucose polymer is a dextrin or a mixture of dextrins, and optionally salts thereof, e.g. poly α-1,4 glucose dextrin.

The glucose polymer or mixtures thereof used in this invention can be produced by hydrolysis of starch in known manner, followed by treatment of the mixture of glucose polymers so obtained in order to remove some or all of the glucose polymers of lower molecular weight.

Preferably, a glucose polymer mixture is prepared by the process described in European Patent Application No. 0 115 911 and removal of lower molecular weight polymers is then effected by known fractionation techniques, such as solvent fractionation, or separation of the polymers with the aid of permeable membranes of appropriate cut-off characteristics.

In general, the fractionation techniques employed are such as to remove mainly the lower molecular weight polymers. If desired, however, they may also be used to remove very high molecular weight polymers if this is found to be necessary in order to ensure that all of the final mixture of glucose polymers is sufficiently water-soluble for there to be no tendency for higher molecular weight polymers to precipitate from solution on standing.

The pharmaceutical composition of the invention may be utilised in the treatment or alleviation of a wide range of yeast and yeast-like fungal infections including, candidiasis, such as Candida albicans. The pharmaceutical composition of the invention may also be advantageous in the treatment or alleviation of fungal and/or protozoal infections resulting from the Human Immunodeficiency Virus (HIV). Such fungal infections include, for example, Pneumocystis carinii and Leishmania Spp. Thus composition of the invention may be useful in the treatment of related diseases, such as Pneumocystis carinii pneumonia (PCP) and/or leishmaniases.

In a further alternative embodiment the pharmaceutical formulation of the invention may be useful in the treatment or alleviation of protozoal infections.

Therefore, according to a further feature of the invention we provide a method of treating a yeast, fungal or protozoal disease which comprises the administering of a therapeutically effective amount of a pharmaceutical composition as hereinbefore described to a patient suffering from such a disorder.

In one embodiment of the invention we provide a method of treating candidiasis which comprises administering of a therapeutic amount of a pharmaceutical composition as hereinbefore described.

In a further embodiment we provide a method of treating a Pneumocystis carinii infection, e.g. Pneumocystis carinii pneumonia (PCP), which comprises administering a therapeutic amount of a pharmaceutical as hereinbefore described.

In a yet further embodiment we provide a method of treating a Leishmania infection, e.g. leishmaniases, which comprises administering a therapeutic amount of a pharmaceutical as hereinbefore described.

The compounds and formulations of the invention are advantageous in that, *inter alia*, they are useful in the treatment or alleviation of yeast, fungal and/or protozoal infections in mammals. Furthermore, the compounds of the invention may target the reticuloendothelial system in mammals. It is a particular advantage of the compounds and formulations of the invention that they have improved solubility e.g. water solubility when compared to the free antifungal agent. Thus, it is an especially advantageous feature of the present invention that a polyene antibiotic therapy can be achieved with improved solubility in water.

Therefore according to a further feature of the invention we provide a polyene antibiotic derivative characterised in that the polyene antibiotic derivative has a solubility in water of from 0.1 mg/ml to 1 mg/ml, preferably 0.1 mg/ml to 0.5 mg/ml and especially 0.1 mg/ ml to 0.4 mg/ml. Amphotericin is known to be insoluble in water at room temperature and substantially neutral pH, i.e. pH 6 to 7. The term insoluble has been defined as 1 part requiring 10,000 parts of solvent to solubilise it. Thus a solubility of less than 100 ppm would be considered to be insoluble. Thus we especially provide a water soluble form of amphotericin, e.g. amphotericin B. We especially provide a form of amphotericin which has a water solubility of at least 1000 ppm at room temperature and substantially neutral pH.

Thus we further provide an aqueous solution of an antifungal agent characterised in that the solution comprises from 0.1 mg/ml to 1 mg/ml, preferably 0.1 mg/ml to 0.5 mg/ml and especially 0.1 mg/ ml to 0.4 mg/ml. We especially provide a solution as hereinbefore described in which the antifungal agent is a dextrin amphotericin compound.

The actual amount of antifungal agent, e.g. amphotericin, in the compound of the invention may vary, depending, *inter alia*, upon the degree of dicarboxylation of the dextrin. Thus, for example the antifungal agent content of the conjugate may be from 0.001 to 25% w/w, preferably from 0.1 to 20% w/w.

In the treatment of yeast, fungal and/or protozoal infections the pharmaceutical composition may be administered in a variety of ways including but not limited to an intravenous infusion, injection or by way of intraperitoneal administration. The most preferred method of administration is by infusion. Accordingly, to a further feature of the invention we provide a composition for intraperitoneal administration containing a pharmaceutical composition as hereinbefore described.

For use in administration to the peritoneal cavity the compositions of the present invention may be in the form of a solution, e.g. an aqueous solution, the

concentration of the composition therein and the administered volume which is selected according to the nature of the treatment involved and the needs of the patient. The solution of composition may contain electrolytes and / or a glucose polymer in a manner similar to that described in UK Patent No. 2207050 which is incorporated herein by reference.

The mode of use of the intravenous infusion solutions according to the invention is similar to that of known solutions.

Alternatively, the pharmaceutical composition may be administered by inhalation. Such a method of administration is especially suitable in, for example, the treatment of Pneumocystis carinii infections and PCP in particular.

Thus, the pharmaceutical composition can be administered by way of an inhaler, e.g. a metered dose inhaler or a dry powder inhaler, an insufflator or nebuliser, or any other conventionally known methods of administering inhalable medicaments.

When administered by way of inhalation the pharmaceutical composition may be in the form of a pressurised aerosol. Thus, according to a further feature of the invention we provide a pharmaceutical formulation suitable for administration by way of a pressurised aerosol comprising a pharmaceutical composition as hereinbefore described in admixture with at least a suitable propellant and optionally with a surfactant or a mixture of surfactants. The propellant is preferably a non-CFC propellant, such as a hydrofluoroalkane (HFA). Any conventionally known HFA propellant may be used, however, HFAs which may be mentioned include a fluoroalkane such as a fluoromethane or a fluoroethane or a mixture of fluoroalkanes. Such fluoroalkanes include, but are not limited to, trichlorofluoromethane, diichlorodifluoromethane, 1,2-dichlorotetrafluorethane, trichlorotrifluoroethane and chloropentafluoroethane, HFA 227 or HFA 134 (1,1,1,2-tetrafluoroethane). The amount of propellant present may vary, but generally the pharmaceutical composition to propellant ratio will be from 1 to 300 to 1 to 5. Mixtures of propellants may be

used, for example, a mixture of HFA 134 and HFA 227. The aerosol composition of the invention may be present as a solution or a suspension of the active ingredient with a propellant.

The pressurised aerosol formulation of the invention may be administered in any conventionally known inhalation apparatus.

In another embodiment the pharmaceutical composition may be administered as an inhalable dry powder formulation. The composition may be administered with or without an adjuvant, diluent or carrier. However, according to the invention we provide a pharmaceutical formulation suitable for administration by way of a dry powder inhaler comprising a pharmaceutical composition as hereinbefore described in admixture with a suitable adjuvant, diluent or carrier. Any conventionally used ingredients in dry powder formulations may be used, such as sugars, these include, but are not limited to, dextran and lactose, e.g. crystalline lactose. Preferably, when a carrier is used the pharmaceutical composition to carrier ratio is from 0.01:50 to 1:1.

The dry powder formulation of the invention may be administered in any conventionally known inhalation apparatus. However, preferred apparatus are those commercially available as CLICKHALER (described in International Patent Application No. WO 92/00771) and/or TECHNOHALER (described in International Patent Application No. WO 93/16748).

Alternatively, the inhalable formulation of the invention may be administered by way of a conventional nebuliser. A suitable nebuliser formulation consists of a sterile solution of the drugs in water optionally containing surfactant or a pharmaceutically acceptable co-solvent or a sterile solution containing finely divided suspended drug. The solution or suspension may be nebulised by an air jet, dropping onto an ultrasonic vibrating plate, forcing through small orifices or other known types of nebuliser, including unit-dose nebulisers such as Respimat (from Boehringer Ingelheim), AERx™ (from Aradigm), and AeroDose (from Aerogen).

For inhalation therapy the pharmaceutical composition is preferably micronised. The particle size of the polymer may vary. However, it is preferred that the polymer will have a particle size of 10 microns or less.

For use in the treatment of yeast, fungal and/or protozoal infections the compositions of the present invention may be in the form of an aqueous solution or may be present as a dry powder for making up into a solution by a physician. The amount of the compound of the invention which is present in the composition may vary, and generally may be from 0.001 to 25% w/w, preferably 0.1% to 20% w/w.

The dosage of pharmaceutical composition administered to a patient may vary depending, *inter alia*, upon the nature and severity of the disorder being treated and the method of administration. Generally, the amount of the pharmaceutical composition administered is preferably in the range of from 0.1 mg to 500 mg from one to four times daily.

We also provide the use of a polyene antibiotic in the manufacture of a compound or a pharmaceutical composition as hereinbefore described.

We especially provide the use of amphotericin in the manufacture of a compound or a pharmaceutical composition as hereinbefore described.

In a yet further aspect of the invention we provide the use of a glucose polymer as hereinbefore described in the manufacture of a compound of the invention.

Thus, the dextrin-amphotericin conjugate of the invention is advantageous, *inter alia*, in that it provides better targeting; reduced side effects; a variety of routes of administration (e.g. intraperitoneal, inhalational); reduced cost and lower amounts of active drug required.

We further provide a method of manufacturing a compound of formula I as hereinbefore described which comprises reacting a dicarboxylated glucose polymer or a derivative thereof with an antifungal agent.

The reaction may be carried out under conventional conditions known *per se*. In particular methods conventionally used in peptide syntheses may be employed. Thus, a dicarboxylated, e.g. succinoylated, dextrin, or a salt thereof may be derivatised prior to reaction with the antifungal agent. Examples of such derivatives include, but are not limited to an acid halide, e.g. acid chloride; acid azide; mixed anhydride; or carbodiimide. A carbodiimide derivative is preferred, for example a carbodiimidazole derivative.

The dicarboxylated glucose polymers and derivatives thereof are novel *per se*. Thus according to a yet further feature of the invention we provide a dicarboxylated glucose polymer, or derivative thereof, for use as an intermediate in the manufacture of a compound of formula I. We especially provide a carbodiimidazole derivative of a dicarboxylated glucose polymer for use as an intermediate in the manufacture of a compound of formula I.

By the term dicarboxylated glucose polymer we mean a glucose polymer which has been reacted with a dicarboxylic acid. Any dicarboxylic acid may be used, thus the dicarboxylic acid may be of the general formula:

$$HOOC(CH_2)_n COOH$$

where n is an integer from 1 to 12

Alternatively, the dicarboxylic acid may be an unsaturated dicarboxylic acid. Specific dicarboxylic acids which may be mentioned include oxalic acid, malonic acid, succinic acid, glutaric acid, adipic acid, pimelic acid, maleic acid and fumaric

acid. The saturated dicarboxylic acids are preferred and succinic acid the most preferred.

The invention will now be described by way of example only.

Example 1

Synthesis of dextrin-Amphotericin B and dextrin sulphate-Amphotericin B

Amphotericin B was conjugated to dextrin (0.9mol%) and dextrin sulphate (1mol%). The method for succinoylating dextrin and dextrin sulphate has been described elsewhere (PCT/GB00/02216).

In brief, dextrin (1g) was dissolved in DMF (10ml). Succinic anhydride (62.5mg, $6.2x10^{-4}$ mol) was added to DMAP (28.5mg) to promote acylation reactions. The mixture was purged with nitrogen and poured rapidly on to diethyl ether and stored overnight. The ether was removed by filtration and remaining solids dissolved in the minimum amount of distilled water and dialysed before being freeze dried.

Succinoylated dextrin (51mg, 1 x 10^{-6} mol) and dextrin sulphate (2.04 x 10^{-6} mol) were each dissolved in 10ml of DMF, to each flask carbonyl diimidazole (CDI) (10.4mg,) was added and left for one hour stirring at room temperature. After this time amphotericin B (3mg, ~1 mol %) was added to each flask and left stirring for 24 hours. The products were then dialysed against deionised water (Mw cut-off 12-14kdA) and freeze dried. The chemical structure of the final product is shown in Figure 15.

Results

The amount of amphotericin B conjugated to the polymers was determined by UV-VIS spectrometry and calibration curve. FTIR also confirmed the presence of

amphotericin B. These reactions were repeated using dextrin and dextrin sulphate with higher degrees of succinoylation to increase the amphotericin B content. Dextrin was succinoylated to levels of 10 and 34.6mol% and dextrin sulphate succinoylated to levels of 14 and 24mol%.

Example 2

Solubility of dextrin-Amphotericin B and dextrin sulphate-Amphotericin B

To test solubility the conjugates were dissolved in water and their solubility compared with that of free amphotericin B. Amphotericin B solutions in water (1, 0.5, 0.25, 0.125, 0.065 and 0.0313 mg/ml Amphotericin B equivalent) were compared with dextrin-Amphotericin B concentrations of 0.4, 0.16 and 0.1 mg/ml Amphotericin B equivalent and dextrin sulphate-Amphotericin B concentrations of 0.014 and 0.009 mg/ml Amphotericin B equivalent.

Results

The conjugates were found to be soluble in water at these concentrations. Amphotericin B is almost totally insoluble in water. In comparison the polymer conjugates were shown to have a ten fold increase in solubility when compared to amphotericin B at the same concentrations.

Example 3

Red Blood Cell Lysis Assay of Amphotericin B Conjugates

For the biological in vitro studies reported here dextrin-Amphotericin B had a drug content of 0.006wt% and dextrin sulphate-Amphotericin B a drug content of 0.014 wt%.

An adult rat was killed and blood quickly removed by cardiac puncture. Blood was placed in a lithium/heparinised tube on ice. PBS was then added and the diluted blood centrifuged three times with the supernatant being removed each time.

A 96-well plate was prepared with samples (100µl n=4) of the appropriate control, polymer or polymer-conjugate. Concentration ranges used for the controls (dextrin, dextrin-sulphate, PBS, Fungizone, DMSO, dextran and polyethyleneimine) were in the range of 0-10mg/ml.

Dextrin-Amphotericin B and dextrin sulphate-Amphotericin B were dissolved in DMSO before being diluted to the appropriate concentration in buffer. Dextrin-amphotericin B concentrations were made in the range 0-2.5µg/ml of Amphotericin B equivalent, dextrin sulphate-Amphotericin B in the range 0-14µg/ml Amphotericin B equivalent, and Amphotericin B concentrations in the range 0-14µg/ml.

Results

The ability of compounds to release haemoglobin from RBC is used to assess the possible adverse effects of intravenous administrations, as the blood is the first compartment in the body that substances will meet. After 1 hour (Figure 16) and 24 hour incubation times both amphotericin B and Fungizone showed a classic sigmoidal response. All the controls (dextrin, dextran, dextrin sulphate, DMSO and polyethyleimine) showed no significant lysis up to a concentration of 10mg/ml at 1 hour. At 24 hours there was no difference in the haemoglobin release, except polyethyleimine displayed a time dependent response.

Using the 1 hour and 24 hour time points the experiment was repeated for dextrin-Amphotericin B and dextrin sulphate-Amphotericin B and compared to equivalent amounts of Amphotericin B (Figures 22 and 23, and Table 1). Amphotericin B was shown to be very haemolytic. The ability of dextrin-Amphotericin B to release haemoglobin was approximately equal to that of Amphotericin B alone at both time

points. In contrast, the Hb_{50} for dextrin sulphate-Amphotericin B was 11 and 8 μg/ml at 1 and 24 hours, respectively. This measurement of haemoglobin release gives a quantitative measurement of gross membrane damage, indicating that the dextrin-sulphate-Amphotericin B results in less membrane damage than Amphotericin B for the same equivalent dose.

Table 1 Haemolytic activity

Compound	Hb_{50} – 1 hour (μg/ml)	Hb_{50} – 24 hour (μg/ml)
Amphotericin B	0.01	0.06
dextrin sulphate-Amphotericin B	11.0	8.0
dextrin-Amphotericin B	not detectable	not detectable

Example 4

Scanning Electron Microscopy (SEM)

This is used to examine the polymer-conjugate interactions with the red blood cells (RBCs), to investigate changes in cell morphology following exposure to polymer, drug or polymer conjugate. RBCs were isolated from adult male rats and incubated at various concentrations. After 24h the preparations were transferred to a microfuge tube and pelleted by centrifugation and supernatant removed. Glutaraldehyde in PBS was added to fix the cells, followed by osmium tetroxide and cells were then dehydrated over time with increasing concentrations of ethanol. At the last drying step hexamethyl disilazine was added: the cells were then placed on a SEM platform using carbon cement and examined using SEM. The concentrations used for the SEM were PBS (control), dextrin and dextrin sulphate (5mg/ml), Amphotericin B at EC_{50}, dextrin-Amphotericin B at EC_{50} (0.5mg/ml) and dextrin sulphate-Amphotericin B (Figure 18) at EC_{50} (0.98mg/ml).

Results

Electron microscopy of RBCs incubated with PBS control for 24 hour revealed cells to be mainly biconcave discs with some appearing to be more spherical (Figure 28). Cells treated with Amphotericin B were no longer identifiable as they appeared as a large mass (Figure 25). Dextrin and dextrin sulphate treated cells (Figures 24 and 27) were comparable with the control cells, which displayed protrusions in the surrounding space (possibly caused by calcium deposits). Dextrin–Amphotericin B treated cells displayed no morphological abnormalities compared to the control cells (Figure 29). RBCs incubated with dextrin sulphate-Amphotericin B (Figure 26) appeared to have congregated with a slightly sponge-like surface, although not to the extent as those treated with Amphotericin B alone. These results indicate that, although dextrin sulphate-Amphotericin B was shown to be moderately haemolytic, treatment with dextrin sulphate-Amphotericin B caused less severe membrane damage than Amphotericin B alone. This may be due to Amphotericin B complexing with the membrane sterols (in particular cholesterol) and the subsequent formation of the pores.

Example 5

In Vitro Cytotoxicity of dextrin-Amphotericin B and dextrin-sulphate-Amphotericin B against B16F10 murine melanoma cell line

Standard cell culture protocols were used to establish the cytotoxic profiles of dextrin-Amphotericin B, dextrin sulphate-Amphotericin B, dextrin and dextrin sulphate. The standard MTT assay is a rapid colormetric assay for assessing cell viablity. The basis of this assay is that viable cells are able to reduce the water soluble tetrazonium dye MTT, to a water insoluble coloured formazan salt. Prior to this experiment a cell growth curve was constructed for the B16F10 cells by titrating the absorbance against time (days). The growth curve confirmed that the cells used in each study were always in the exponential phase of cell growth. A 96-well microtitre

plates were seeded with a suspension of 10^4 cells per well of B16F10 murine melanoma cells in RPMI 1640 media containing 10% foetal calf serum. The cells were allowed to incubate for 24 hours at 37°C/5 % CO_2.

The growth media was carefully removed and 100µl of controls and polymers were dissolved in growth media (n=8). Dextrin-Amphotericin B concentrations were made in the range of 0-2.5µg/ml of Amphotericin B equivalent, and dextrin sulphate-Amphotericin B was in the range of 0-14µg/ml Amphotericin B equivalent.

The plates were then incubated as above for a further 67 hours and 20µl of MTT was added to each well. The plates were then incubated for a further 5 hours when all the media was carefully removed and 100µl of DMSO was added to each well. Crystals of the blue tetrazolium dye were allowed to dissolve into the DMSO for a few minutes when the absorbance at 550nm of each well was read in a microtitre plate reader.

The absorbance values were then converted to % cell viability based on value for untreated cells. Finally cell viability was plotted against drug concentration.

Results

The MTT assay assesses the ability of compounds to decrease cell viability. B16F10 cells were selected as they are a well characterised mammalian cell-line in which Amphotericin B is active, although to a lesser extent than fungal cells.

The MTT assay with incubation of dextran, dextrin, and dextrin sulphate caused very little decrease in cell viability at the highest concentrations used (5mg/ml). Poly-L-lysine was a positive control with an IC_{50} of approximately 0.04mg/ml. Amphotericin B also caused significant decrease in cell viability, in an exponential manner, IC_{50} value of 80µg/ml (Figure 19).

Both the dextrin-Amphotericin B and the dextrin sulphate-Amphotericin B caused a greater decrease in cell viability than equivalent quantities of Amphotericin B. Dextrin–Amphotericin B conjugate caused an initial decrease in cell viability to approximately 75% at a dose of 0.4μg/ml (0.2μg/ml Amphotericin B equivalent), which then remained at that level even at the highest concentration used (Figure 20). Dextrin sulphate-Amphotericin B was significantly more toxic to the B16F10 cells than equivalent quantities of Amphotericin B, with an IC_{50} value of approximately 11μg/ml Amphotericin B equivalent, which is approximately 8 times lower than Amphotericin B alone (Figure 21).

Table 2

Activity of amphotericin B formulations
against *Leishmania donovani* L82 in BALB/c mice.

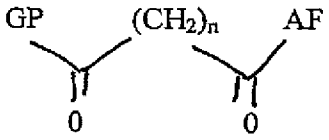
	Dosing regimen	% inhibition ± S.E.M	ED_{50} (ED_{90})
dextrin succinoyl amphotericin B	12.5mg/kg i.p. x 3	98.1 ± 0.9	**6.1 (9.12)**
	5mg/kg i.p. x 3	25.32 ± 4.23	
	1mg/kg i.p. x 3	18.42 ± 6.2	
dextrin-2-sulphate-succinoyl-amphotericin B	12.5mg/kg i.p. x 3	99.45 ± 0.1	**1.34 (3.8)**
	5mg/kg i.p. x 3	94.04 ± 0.6	
	1mg/kg i.p. x 3	35.18 ± 17.8	
dextrin succinoyl (≡25mg/kg)	i.p. x 3	32.32 ± 4.2	
dextrin-2-sulphate succinoyl (≡25mg/kg)	i.p. x 3	19.21 ± 4.2	
AmBisome[1]	5mg/kg i.v. x 3	100	**<0.2 (0.5)**
	1mg/kg i.v. x 3	99.95 ± 0.1	
	0.2mg/kg i.v. x 3	98.5 ± 0.3	
Pentostam[2]	15mgSb^{5+}/kg s.c. x 5	31.72 ± 8.4	

[1] Intravenous liposomal amphotericin B formulation

[2] Sodium stibogluconate (pentavalent antimony)

CLAIMS

1. A compound of the general formula I,



I

wherein GP is a glucose polymer, or a mixture of glucose polymers, and optionally salts thereof;
AF is an antifungal agent; and
n is an integer from 1 to 12.

2. A compound according to Claim 1 characterised in that the antifungal agent possesses at last one free amine group.

3. A compound according to Claim 1 characterised in that the antifungal agent is a polyene antibiotic.

4. A compound according to Claim 3 characterised in that the polyene antibiotic is selected from amphotericin and nystatin.

5. A compound according to Claim 4 characterised in that the polyene antibiotic is amphotericin.

6. A compound according to Claim 5 characterised in that the amphotericin is amphotericin B.

7. A compound according to Claim 1 characterised in that the glucose polymer is a dextrin, or a mixture of dextrins, and optionally salts thereof.

8. A compound according to Claim 7 characterised in that the glucose polymer, or a mixture of glucose polymers, and optionally salts thereof, is selected from those polymers described in European Patent Application No. 0 115 911 and/or No. 0 153 164.

9. A compound according to Claim 7 characterised in that the glucose polymer is a poly α-1,4 glucose dextrin or a salt thereof.

10. A compound according to Claim 7 characterised in that the salt of the glucose polymer or a mixture of glucose polymers is an anionic salt.

11. A compound according to Claim 10 characterised in that the anionic salt is the sulphate.

12. A compound according to Claim 7 characterised in that the glucose polymer which substantially comprises dextrin-2-sulphate.

13. A pharmaceutical composition comprising an effective amount of a complex of an antifungal agent and a glucose polymer or a mixture of glucose polymers and, optionally, salts thereof.

14. A method of treating, alleviating or preventing a yeast, protozoal and/or fungal infection which comprises the administering of a therapeutically effective amount of a compound according to Claim 1 or a composition according to Claim 13 to a patient suffering from such a disorder.

15. A method according to Claim 14 characterised in that the fungal disease candidiasis.

16. A method according to Claim 14 characterised in that the yeast and/or protozoal infection is caused by Pneumocystis carinii

17. A method according to Claim 14 characterised in that the yeast and/or protozoal infection is caused by Leishmania.

18. A method according to Claim 14 characterised in that the composition is administered as a solution.

19. A method according to a claim 18 characterised in that the solution is administered intravenously, intramuscularly or intraperitoneally.

20. A method according to claim 19 characterised in that the solution is administered intraperitoneally.

21. A method according to Claim 14 characterised in that the amount of the compound administered in the range of from 0.1 mg to 500 mg from one to four times daily.

22. A pharmaceutical composition according to claim 13 characterised in that the composition is suitable for administration as a pressurised aerosol and is in admixture with a suitable propellant.

23. A pharmaceutical composition according to claim 13 characterised in that the composition is suitable for administration as an inhalable dry powder formulation and is optionally in admixture with a suitable adjuvant, diluent or carrier.

24. A pharmaceutical composition according to Claim 23 characterised in that the particle size of the pharmaceutical composition is 10 microns or less.

25. A pharmaceutical composition according to claim 13 characterised in that the composition is suitable for administration by way of a nebuliser comprising a solution or a suspension of the pharmaceutical composition.

26. A pharmaceutical composition according to Claim 13 characterised in that the amount of the compound of formula 1 in the composition is from 0.001 to 25% w/w.

27. A polyene antibiotic derivative characterised in that the polyene antibiotic derivative has a solubility in water of from 0.1 to 1.0 mg/ml.

28. The use of a polyene antibiotic in the manufacture of a compound according to claim 1 and/or a composition according to claim 13.

29. The use of amphotericin in the manufacture of a compound according to claim 1.

30. The use of amphotericin in the manufacture of a pharmaceutical composition according to claim 13.

31. The use of a glucose polymer in the manufacture of a medicament for the treatment, alleviation or prevention of a yeast, protozoal and/or fungal infection.

32. A method of manufacturing a compound according to claim 1 which comprises reacting a dicarboxylated glucose polymer or a mixture of glucose polymers or a derivative thereof with an antifungal agent.

33. A method according to claim 32 characterised in that the dicarboxylated glucose polymer, or a mixture of glucose polymers, is manufactured by reacting a glucose polymer, or a mixture of glucose polymers with a dicarboxylic acid of the general formula II:

$$H00C\ (CH_2)_n\ C00H \qquad II$$

where n is an integer from 1 to 12.

34. A method according to claim 33 characterised in that the dicarboxylic acid is succinic acid.

35. A dicarboxylated glucose polymer or a derivative thereof for use as an intermediate in the manufacture of a compound of formula I.

36. A dicarboxylated glucose polymer according to claim 35 characterised in that it is a succinoylated dextrin.

37. A dicarboxylated glucose polymer according to claim 36 characterised in that the derivative is a carbodiimidazole derivative.

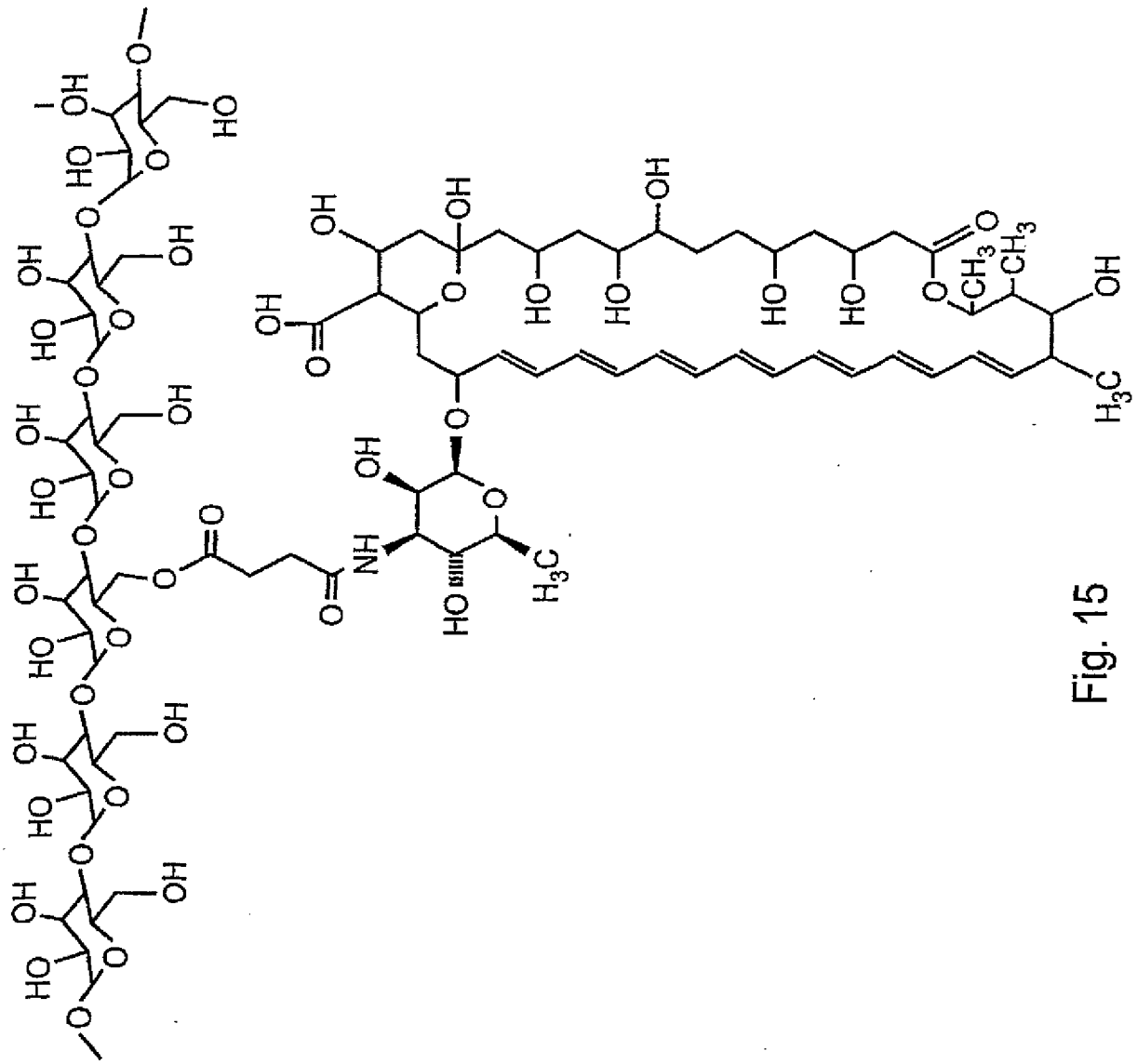


Fig. 15

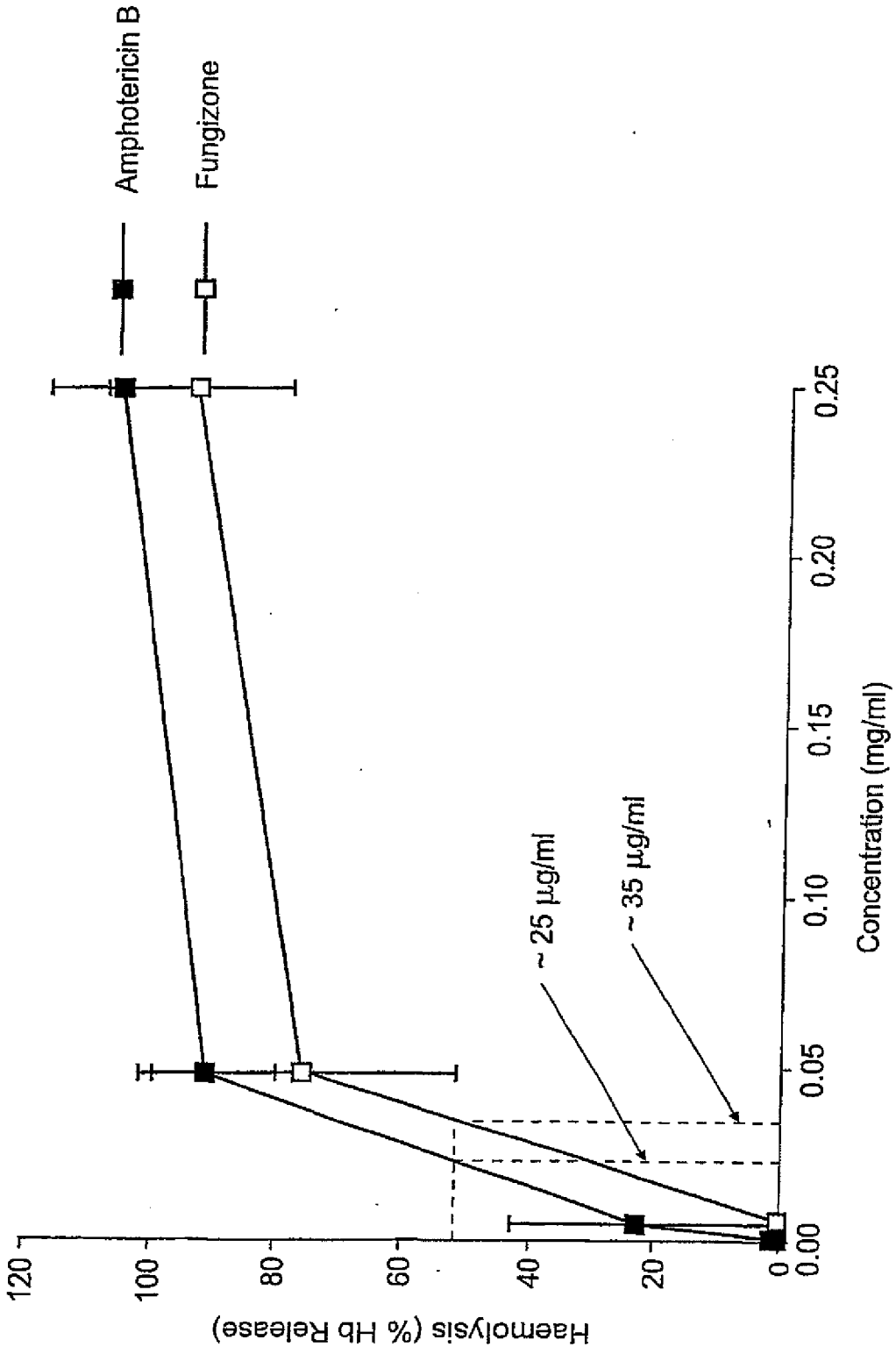


Fig. 16

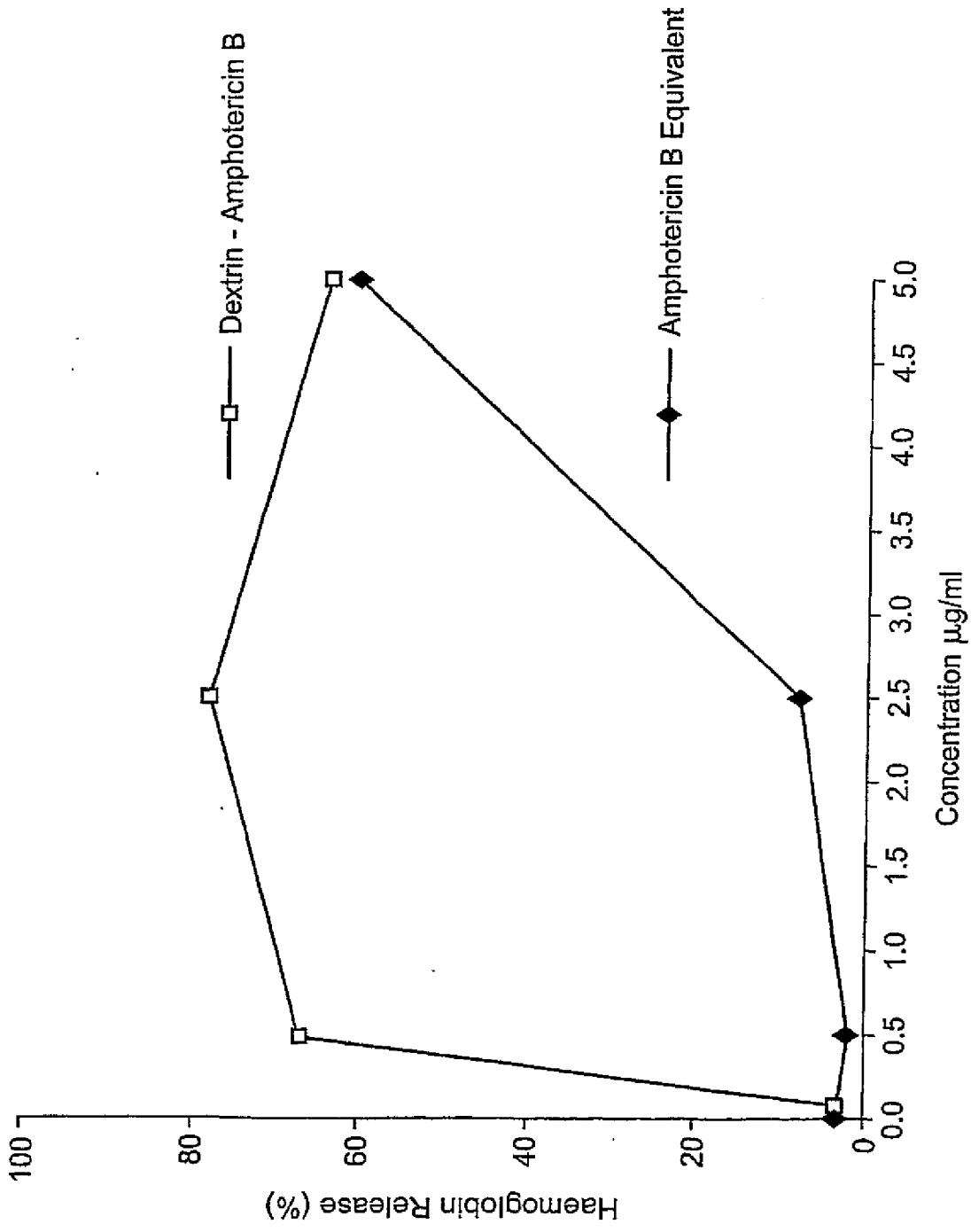


Fig. 17

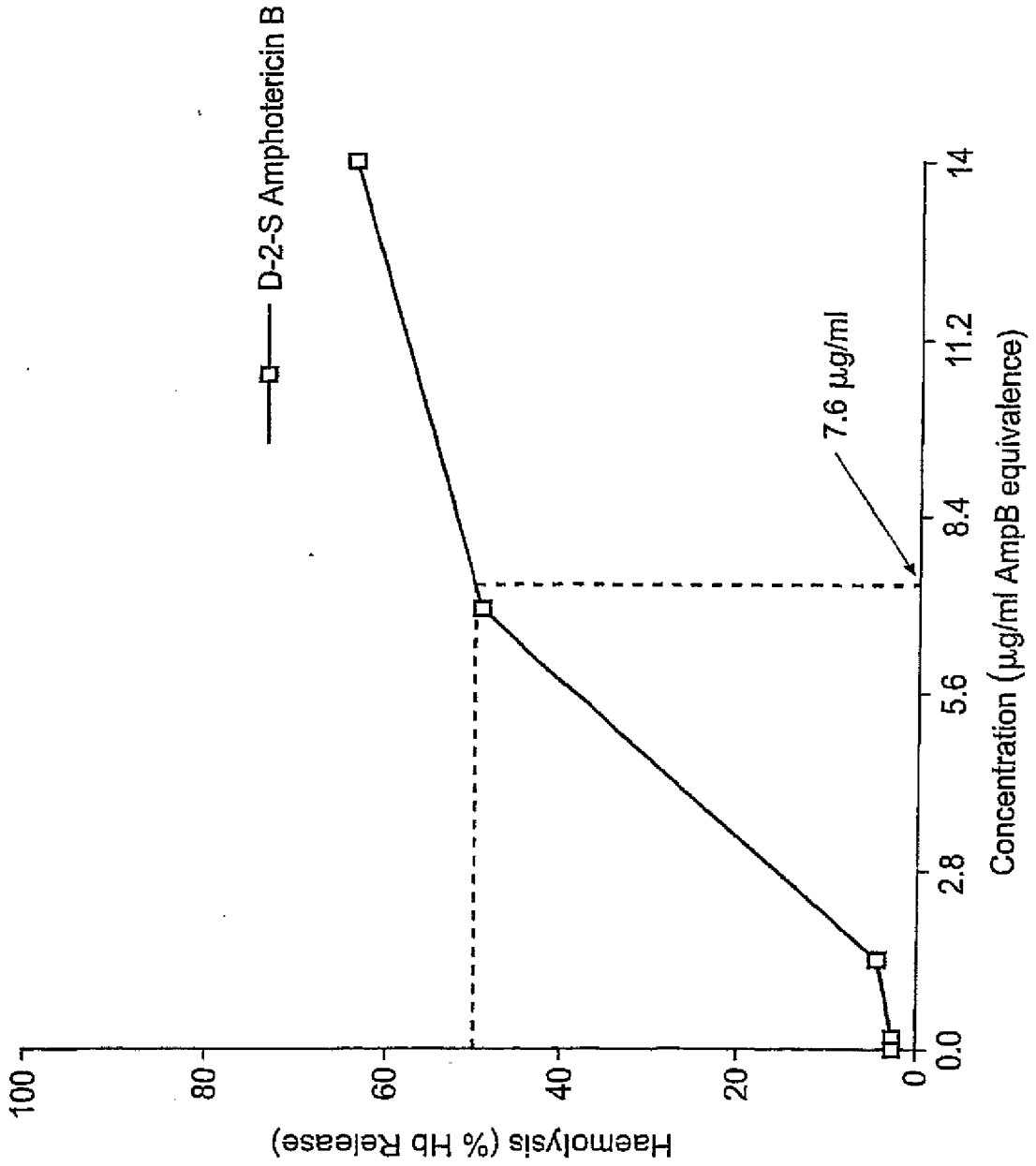


Fig. 18

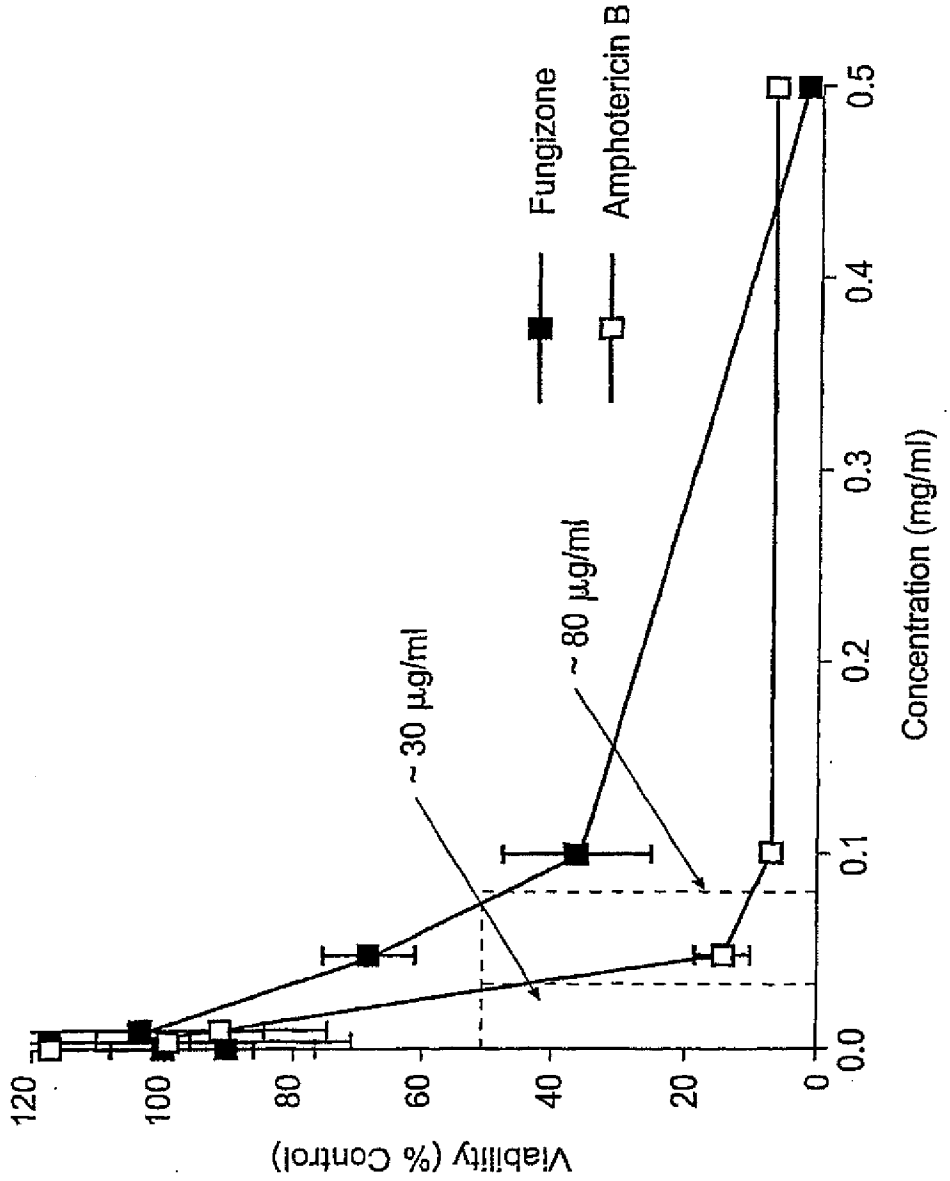


Fig. 19

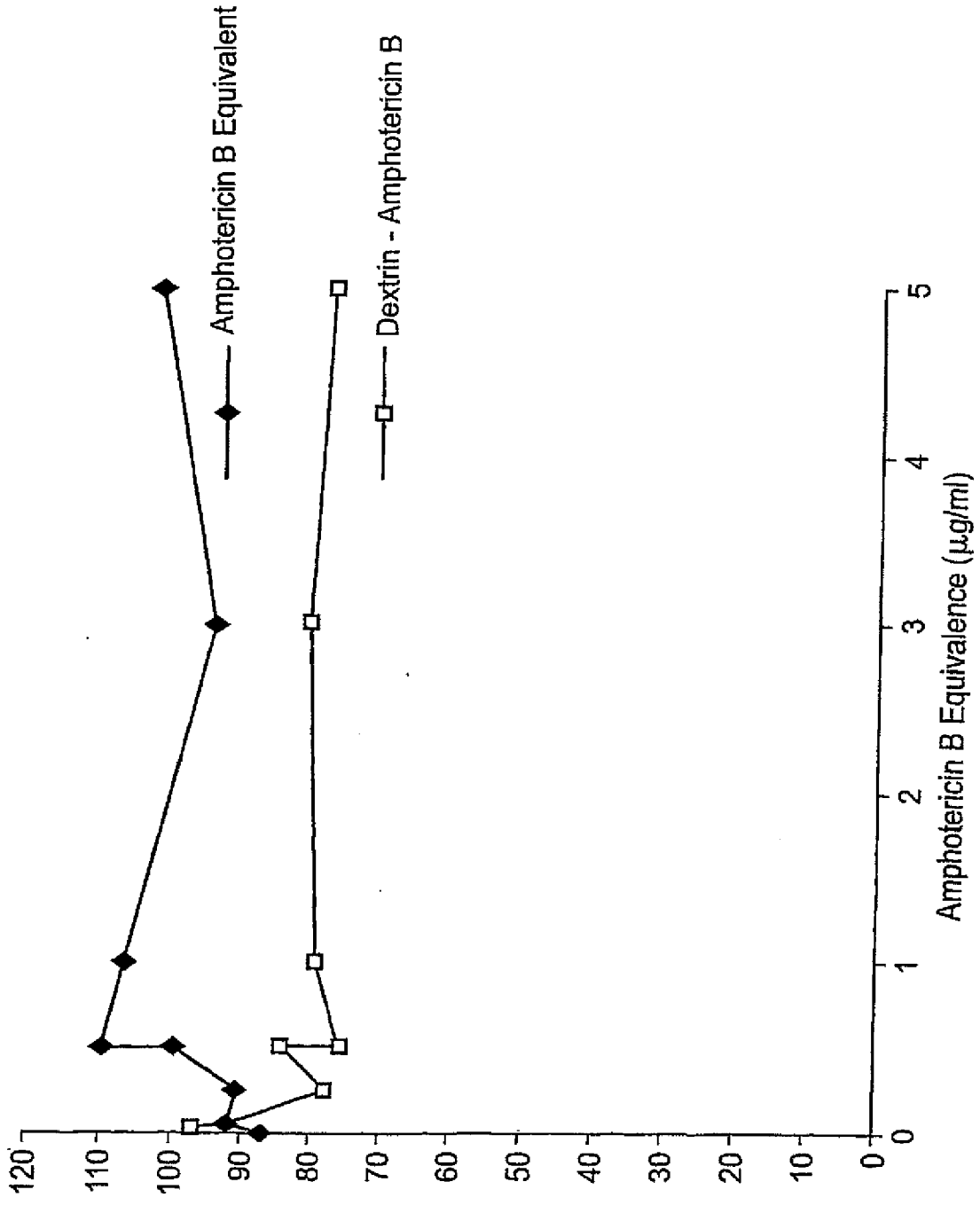


Fig. 20

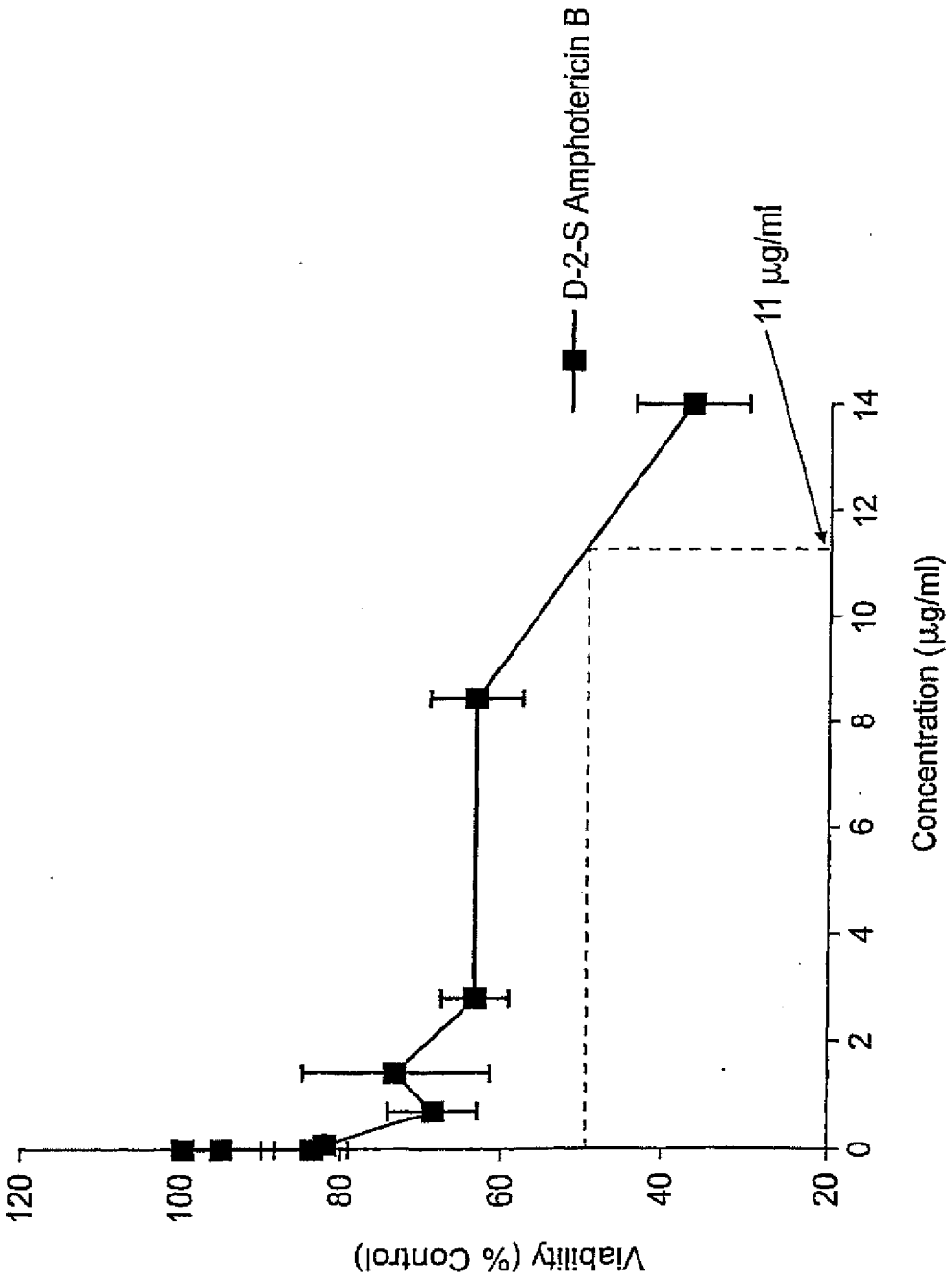


Fig. 21

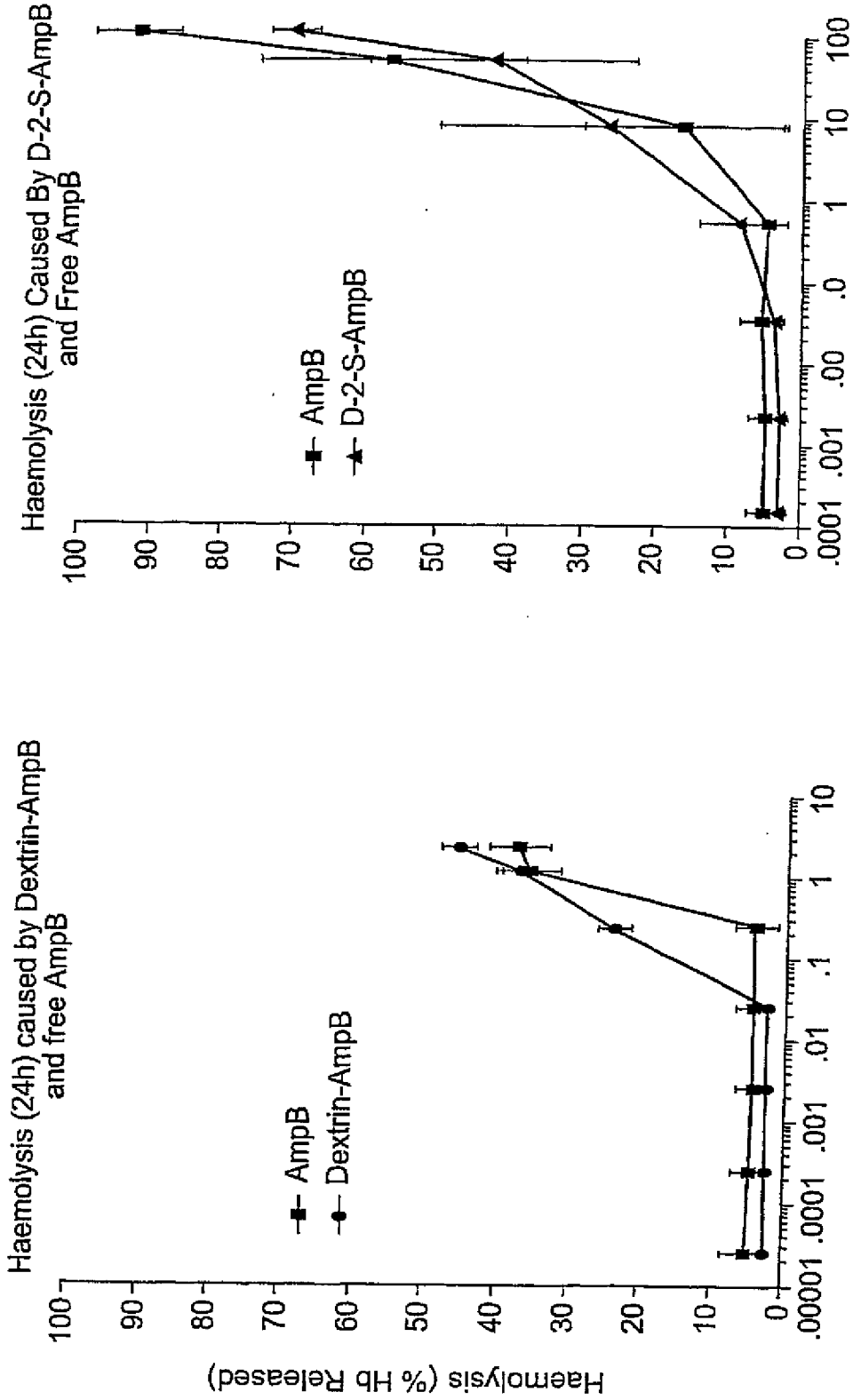
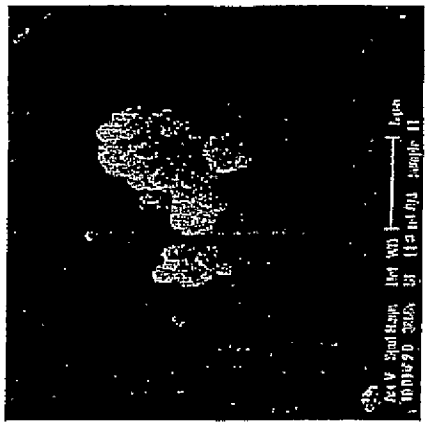


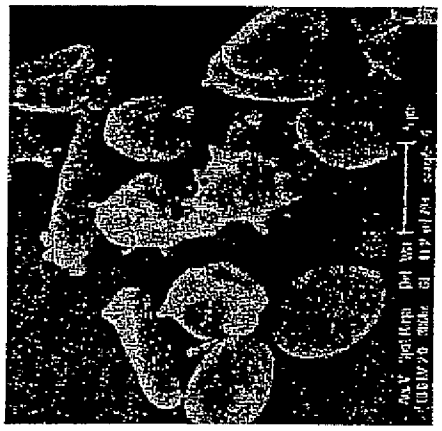
Fig. 22

Fig. 23



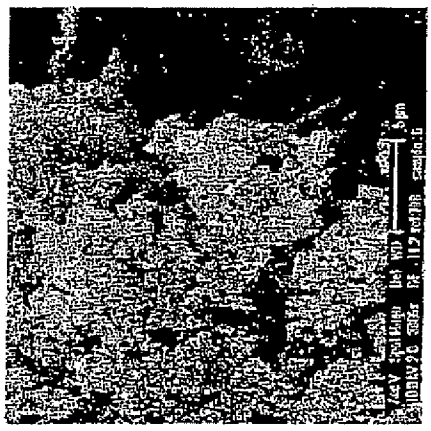
D-2-S-AmpB at EC_{50}

Fig. 26



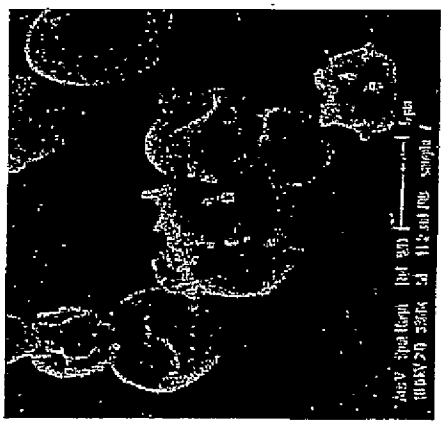
Dextrin- AmpB at 0.5 mg/ml

Fig. 29



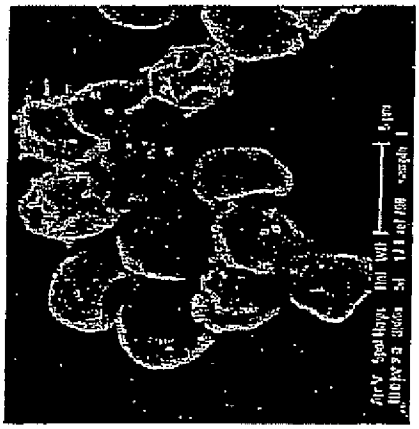
AmpB

Fig. 25



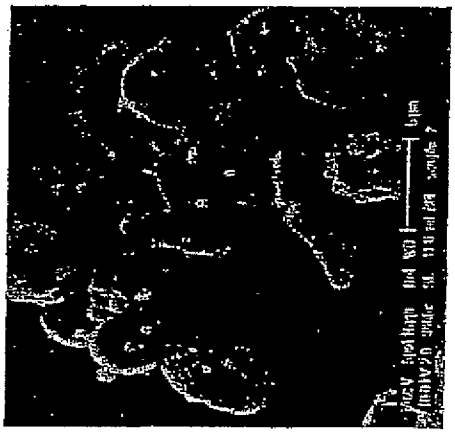
PBS

Fig. 28



Dextrin at 5 mg/ml

Fig. 24



D-2-S at 5 mg/ml

Fig. 27

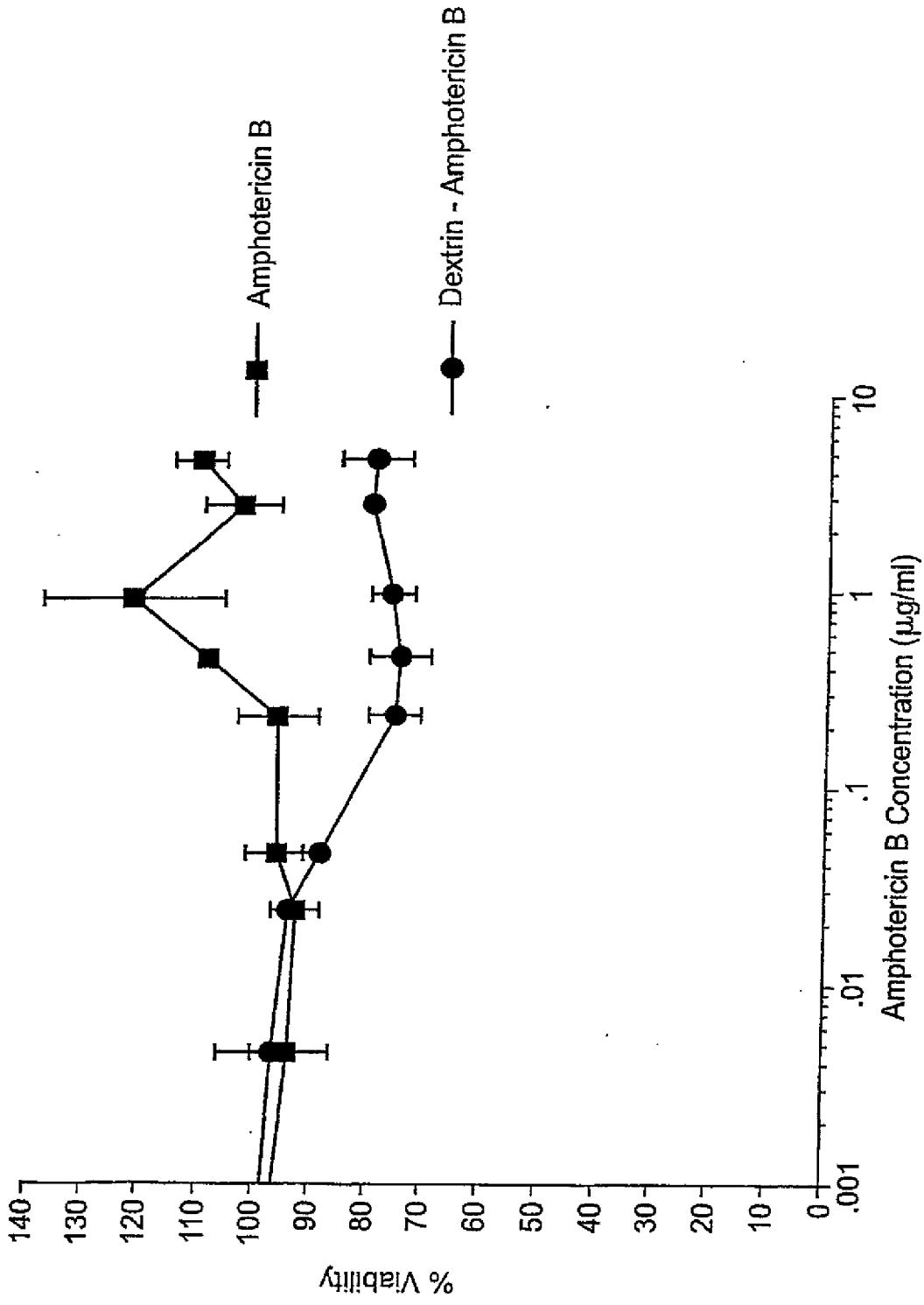


Fig. 30

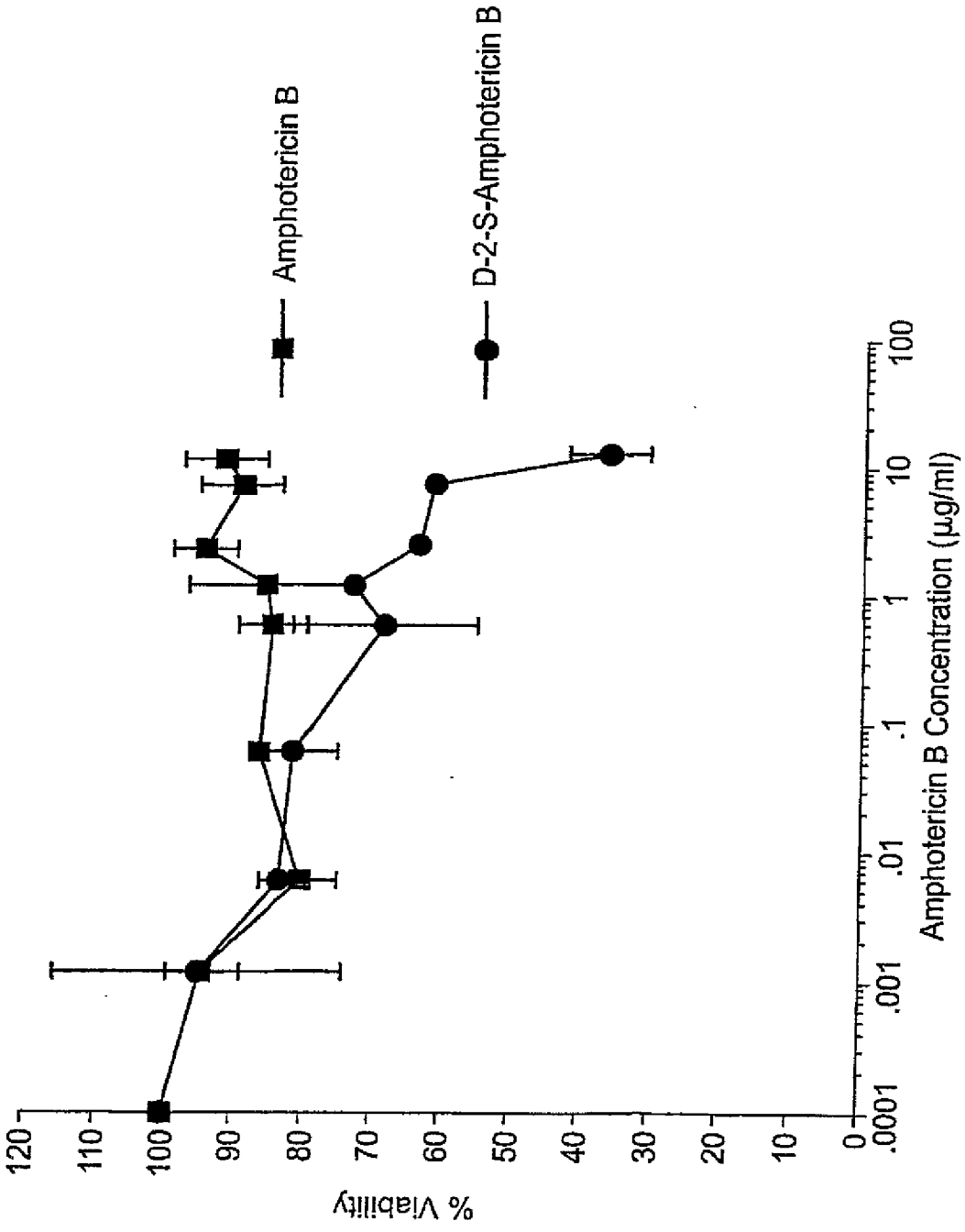


Fig. 31

(12) INTERNATIONAL [redacted]LICATION PUBLISHED UNDER THE PATENT [redacted]PERATION TREATY (PCT)

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(25) Filing Language: English

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(74) Agent: **HARRISON GODDARD FOOTE**; 31 St. Saviourgate, York Y01 8NQ, Leeds LS2 8PA (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

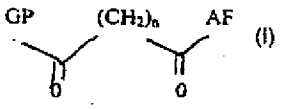
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
13 June 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DEXTRIN-AMPHOTERICIN CONJUGATES AND THEIR METHODS OF PREPARATION



(I)

(57) Abstract: There is described a compound of the general formula (I): wherein GP is a glucose polymer, or a mixture of glucose polymers, and optionally salts thereof; AF is an antifungal agent; and n is an integer from 1 to 12. There is also described compositions containing this compound and methods of treatment relating thereto.

Dextrin- AmpB at 0.5 mg/ml

WO 02/02146 A3

INTERNATIONAL SEARCH REPORT

Interna[redacted]nal Application No
PC[redacted] 01/02886

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GERMAN L A ET AL: "Dextrin-amphotericin B: A potential polymeric anti-infective or antiparasitic agent." JOURNAL OF PHARMACY AND PHARMACOLOGY, vol. 52, no. Supplement, September 2000 (2000-09), page 37 XP001022489 137th British Pharmaceutical Conference;Birmingham, England, UK; September 10-13, 2000 ISSN: 0022-3573 the whole document	1-12,27, 29,31-37
X	WO 98 56424 A (CASSIDY JAMES ;DUNCAN RUTH (GB); GERMAN LISA (GB); HIRST DALE (GB)) 17 December 1998 (1998-12-17)	1,7-12, 31-36
Y	page 4, line 16-26; claims; figures 3A,3B; examples	1-12,27, 29,31-37
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2001	03. 04. 02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Veronese, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YOUNG D H ET AL: "ANTIFUNGAL PROPERTIES OF TAXOL AND VARIOUS ANALOGUES" EXPERIENTIA, BIRKHAUSER VERLAG. BASEL, CH, vol. 48, no. 9, 1992, pages 882-885, XP000929422 ISSN: 0014-4754 the whole document	1,7-12, 32-36
X	DUNCAN R ET AL: "Dextrins as Carriers of Anticancer Agents" PROCEEDINGS OF THE 24TH. INTERNATIONAL SYMPOSIUM ON CONTROLLED RELEASE OF BIOACTIVE MATERIALS. STOCKHOLM, JUNE 15 - 19, 1997, PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM ON CONTROLLED RELEASE OF BIOACTIVE MATERIALS, DEERFIELD, IL., CONTROLLED RELEASE, vol. SYMP. 24, 15 June 1997 (1997-06-15), pages 771-772, XP002078115 ISSN: 1022-0178	35,36
Y	the whole document	1-12,27, 29,31-37
Y	FALK RAMA ET AL: "A novel injectable water-soluble amphotericin B-arabinogalactan conjugate." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 43, no. 8, August 1999 (1999-08), pages 1975-1981, XP002182534 ISSN: 0066-4804 page Y	1-12,27, 29,31-37
Y	WO 90 15628 A (CETUS CORP) 27 December 1990 (1990-12-27) claims 7,21	1-12,27, 29,31-37
A	EP 0 466 038 A (SQUIBB & SONS INC) 15 January 1992 (1992-01-15) the whole document	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 01/02886

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
27 (partial); 1-12, 29, 31-37 (complete

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-12, 27, 29, 31-37 relate to an extremely large number of possible compounds. In the present case it is not possible from formula (I) to determine how and in which position the polymer and the antifungal agent are linked to the spacer; therefore a lack of clarity exists in the sense of art 6 PCT. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds of the general formula I in which the glucose polymer is a dextrin, (in particular 1,4 glucose dextrins) and the antifungal agent is a polyene antibiotic, (in particular amphotericin and nystatin).
Accordingly, the search for claims 35-37 has been restricted to the dextrin derivatives.

Claim 31, which covers the use of any glucose polymer in the manufacture of a medicament for the treatment of fungal infections, is supported only for those parts related to derivatives in which the glucose polymer is conjugated to an antifungal agent as shown in the compounds of formula I in claim 1. The search has therefore only covered this part of the claim.

Moreover, claim 27 relates to a compound defined by reference to a desirable characteristic or property, namely a certain solubility in water. The claim covers all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds of formula (I) with the restrictions which have already indicated above.

Claims 1-12, 27, 29, 31-37 have been searched partially.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 27 (partial); 1-12,29, 31-37 (complete).

   Compounds having the general formula I as shown in claim 1, their method of preparation as well as the intermediate derivatives disclosed in claims 32-37, and their use in relation to the treatment of fungal infections.

2. Claims: 27 (partial); 13-26, 28, 30 (complete).

   Pharmaceutical compositions comprising a complex of an antifungal agent and a glucose polymer.

INTERNATIONAL SEARCH REPORT

[redacted]ion on patent family members

Interr[redacted]al Application No
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